PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of

MASAHIKO TERAKADO, et al.

Docket No: Q87291

Appln. No.: 10/530,249

Group Art Unit: 1609

Filed: April 04, 2005

Examiner: Murray, Jeffrey H

For: LPA RECEPTOR ANTAGONIST

DECLARATION UNDER 37 C.F.R. § 1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Masahiko Terakado, hereby declare and state:

THAT I am a citizen of Japan;

THAT I have received my PhD in Organic Chemistry in 1994 from Tokyo Institute of Technology.

THAT I have been employed by Ono Pharmaceutical Co., Ltd. since 1994, and I study of lysophosphatidic acid at the Research Institute of Ono Pharmaceutical Co., Ltd.;

THAT this declaration is made in support of the above identified U.S. Patent Application and is provided to demonstrate the unexpected or unobvious effect of the present invention;

THAT I am a co-inventor of the present application;

THAT the following experiments (Activity of LPA Receptor Antagonist) were conducted under my direct supervision.

Activity of LPA Receptor Antagonist

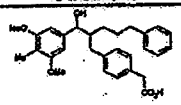
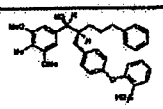
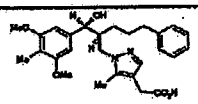
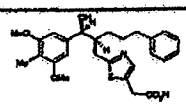
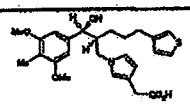
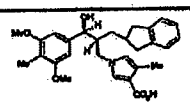
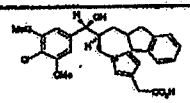
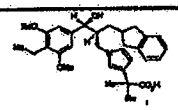
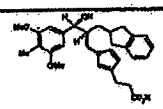
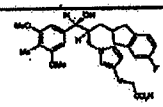
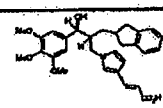
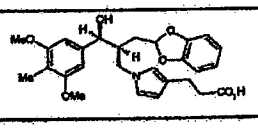
Evaluation of EDG-2 antagonistic activity by monitoring the change of intracellular calcium ion concentration

Evaluation of EDG-2 antagonistic activity was carried out by using Chinese hamster ovary (CHO) cells which overexpressed human EDG-2 gene. Those cells were cultured with Ham's F12 medium (GIBCO BRL company No.11765-047) containing 10% FBS (fetal bovine serum), penicillin/streptomycin and blasticidin (5 μg/ml). At first in oreder to uptake Fura2-AM(Dojindo company No.348-05831) into the cells cells, cells were incubated for 60 minutes at 37 degrees in Fura2-AM(5 μM) solution [Ham's F12 medium containing 10%FBS, 20 mM HEPES buffer (pH 7.4) and 2.5 mM probenecid (Sigma company No.P-8761)]. Next, it was washed with Hanks solution containing HEPES buffer (20 mM, pH 7.4) and probenecid (2.5 mM) once, and immersed into the Hanks solution. Plates were set in fluorescent drug screening system(Hamamatsu photonics company, FDSS-2000) and intracellular calcium ion concentration was measured for 30 seconds with no stimulation and then solution of the compound of the present invention of formula (I) was added. Five minutes after adding thereto LPA (final concentration :100 nM) was added, the increase of intracellular calcium ion concentrations before and after the addition of LPA (excitation wave length: 340 nM and 380 nM; fluorescent wave length: 500 nm) were measured every 3 seconds. The compound of the present invention represented by the formula (I) was dissolved in dimethyl sulfoxide (DMSO), and it was added so that the final concentration became 1nM to 10 μM. 1-oleoyl (18:1)-LPA(Sigma) or 1-linolenoyl (18:3)-LPA was used as LPA. 1-linolenoyl (18:3) -LPA was synthesized and purified in the either way shown below. (i) the way of synthesizing 1-linolenoyl (18:3) -LPA from (18:3) – LPC (linolenoyl (18:3)-lysophosphatidylcholine) (Sedary company) by PLD (phospholipase D), or (ii) the way of synthesizing 18:3-LPC (linolenoyl (18:3)-lysophosphatidylcholine) from 18:3-PC(linolenoyl (18:3)-phosphatidylcholine) (Avanti Polar Lipids) by PLA_2, followed by synthesizing LPA from it by PLD (phospholipase D). EDG-2 antagonistic activity was calculated as an inhibition rate (%) by the following equation, wherein the peak value of LPA (final concentration: 100 nM) in a well into which DMSO containing no test compound represented by the formula (I) was added was regarded as a control value (A), and in the cells treated with the test compound the difference (B) between the value before addition of the test compound and that after the addition was obtained and compared with the control value.

$$\text{Inhibition rate (\%)} = [(A-B) / A] \times 100$$

The IC_{50} value was calculated as a concentration of the compound to be tested which showed 50% inhibition.

The results are shown below.

Structure	IC50 (μM)
	0.61
	0.022
	0.077
	0.028
	0.025
	0.29
	0.078
	0.043
	0.086
	0.13
	0.27
	0.051

I declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: September 5, 2007

Masahiko Terakado
Masahiko TERAKADO